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Archival search for historical atypical scrapie in sheep reveals evidence for mixed infections.

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Summary

Natural scrapie in sheep occurs in classical and atypical forms, which may be distinguished on the basis of the associated neuropathology and properties of the disease-associated prion protein on Western blots. First detected in 1998, atypical scrapie is known to have occurred in UK sheep since the 1980s. However its aetiology remains unclear and is often considered as a sporadic, non-contagious disease unlike classical scrapie which is naturally transmissible. Although atypical scrapie tends to occur in sheep of prion protein (*PRNP)* genotypes that are different from those found predominantly in classical scrapie, there is some overlap so that there are genotypes in which both scrapie forms can occur. In this search for early atypical scrapie cases, we made use of an archive of fixed and frozen sheep samples, from both scrapie affected and healthy animals (~1850 individuals), dating back to the 1960s. Using a selection process based primarily on *PRNP* genotyping but also on contemporaneous records of unusual clinical signs or pathology, candidate sheep samples were screened by Western blot, immunohistochemistry and strain typing methods using tg338 mice. We have identified, from early time points in the archive, three atypical scrapie cases, including one sheep which died in 1972, and two of which show evidence of mixed infection with classical scrapie. Cases with both forms of scrapie in the same animal as recognisable entities, suggest that mixed infections have been around for a long time and may potentially contribute to the variety of scrapie strains.

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Introduction

Natural scrapie in sheep is one of a group of diseases, affecting several mammalian species, known as transmissible spongiform encephalopathies (TSEs) or prion diseases. A hallmark of TSEs is the detection in brain (and sometimes also lymphoreticular tissues) of an abnormal form of the prion protein, known by various short forms including PrP^{Sc} and PrP^{d} to distinguish it from the normal cellular protein PrP or PrP^{C} (Bolton *et al.*, 1982; Hope *et al.*, 1986). PrP^{Sc} is relatively proteinase K (PK) resistant and on Western blots usually has a distinct three banded pattern (the result of differential glycosylation), and particular patterns and sizes of the bands can be used as part of strain-typing of TSEs (Gavier-Widen *et al.*, 2005). Incidence of scrapie is highly dependent on *PRNP* genotype at codons 136,154 and 171 with, for example, $V_{136}R_{154}Q_{171}$/VRQ animals at very high risk of disease, and ARR/ARR and heterozygotes at low risk, [for review see (Goldmann, 2008)].

A different form of ovine TSE, termed Nor98 or atypical scrapie, was discovered in Norway in 1998 (Benestad *et al.*, 2003). It is biologically, neuropathologically and biochemically distinct from classical natural scrapie (Table S1). For example atypical scrapie PrP^{Sc} is less PK resistant than classical scrapie PrP^{Sc} and has more variable pattern on Western blots (WB), including characteristic low molecular mass band(s) variously estimated at ~7-12kDa, not found in classical scrapie (Le Dur *et al.*, 2005). Since 1998, atypical scrapie cases have been identified throughout Europe, including the UK, mainly through active surveillance of asymptomatic sheep (Buschmann *et al.*, 2004; De Bosschere *et al.*, 2004; Nentwig *et al.*, 2007; Orge *et al.*, 2004; Polak *et al.*, 2010). Atypical scrapie tends to occur in older sheep and in animals with *PRNP* genotypes considered to be resistant to classical scrapie (Benestad *et al.*, 2008;

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Saunders *et al.*, 2006) such as those with AHQ and ARR alleles. It is also associated with codon 141, which varies only on the ARQ allele, such that genotypes including the $AF_{141}RQ$ allele are more susceptible than those with $AL_{141}RQ$. There is some overlap of susceptibility however, as some genotypes are found in both atypical and classical scrapie cases, for example VRQ/$AL_{141}RQ$ and $AL_{141}RQ$/$AL_{141}RQ$ (Fediaevsky *et al.*, 2008). Indeed there is evidence for both scrapie forms occurring in a single $AL_{141}RQ$/$AF_{141}RQ$ animal (Mazza *et al.*, 2010).

Incidence of atypical scrapie in the UK is low but consistent. In 2012 and 2013, it was found in ~0.1% of the >18,000 sheep which were tested in abattoir and fallen stock surveys each year (Ortiz-Pelaez & Arnold, 2013). Atypical scrapie is not thought to be naturally transmissible although successful experimental transmissions have been achieved in sheep and transgenic mice, in the latter with similar pathology to that seen in the original sheep (Andreoletti *et al.*, 2011; Le Dur *et al.*, 2005; Simmons *et al.*, 2010; Simmons *et al.*, 2007).

Originally it was not certain whether atypical scrapie was a newly emerging TSE or whether a pre-existing disease had been identified by increased surveillance. Archive searches have found cases in the UK from 1989 (Bruce *et al.*, 2007; Foster *et al.*, 2008) and 1987 (Webb *et al.*, 2009) indicating that it is not a new disease. We took advantage of the Neuropathogensis Unit (NPU) sheep tissue archive (now stored at The Roslin Institute), which has samples dating back to the 1960s, to search for additional early examples of atypical scrapie in order to establish its history as far as possible. It was of particular interest to look for examples of TSE infections with features of both atypical and classical scrapie as the origin of atypical scrapie is

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unknown and one possibility is that it developed from a type of classical scrapie. Here we report evidence for early unusual scrapie cases with individual sheep apparently showing signs of multiple strain infections.

Results

Selection of candidate atypical scrapie cases

We examined two sheep tissue archives (1) sheep (n~350) from throughout the UK and (2) sheep (n~1500) from our own flock (NPU Cheviots). Samples, which varied considerably in quality due to long term storage, were put through a non-rigorous selection process designed to maximise the chances of finding atypical scrapie at early dates but not expected to find every case present. Further details are given in Methods and Fig. S1.

Two UK archive sheep were considered to be candidate atypical scrapie cases. The first, L4824 (AHQ/ARR) was one of a pair of suspect scrapie cases from 1988 from a flock in Scotland, both female Cheviots of unrecorded age. While L4824 had been judged in 1988 to be negative for brain vacuolation in medulla, the companion case (L4823, VRQ/AL_{141}RQ) was diagnosed as classical scrapie based on positive brain vacuolar pathology. Fixed tissue was available for us to carry out immunohistochemistry (IHC) with BG4 antibody and L4824 displayed the disease-related PrP (PrP^d) deposition characteristic of atypical scrapie, with marked labelling in the molecular layer in the cerebellum but very little in obex and basal ganglia (Fig. S2). There was also evidence of microvacuolation in the cerebellum, clearly not spotted in 1988. In contrast L4823 had PrP^d labelling in obex and basal ganglia rather than cerebellum (Fig. S2) which suggested it was classical scrapie.

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The second UK archive candidate atypical scrapie case was H800, a female Poll Dorset sheep (VRQ/AF_{141}RQ *PRNP* genotype) which died in 1977. There is no further recorded information about this animal and no fixed tissue for immunohistochemistry. It was discovered by Western blot (WB) analysis, detailed below.

All three sheep L4824, L4823 and H800 were positive by WB for PrP^{Sc}. L4824 displayed a low molecular mass PrP^{Sc} band (Fig.1a), estimated on this WB at 7-9kDa, indicative of atypical scrapie and recognised by P4 but not 6H4 antibodies (Fig. 1a and c), similar to cases previously described by ourselves and others (Foster *et al.*, 2008). L4823 had the pattern of PrP^{Sc} expected from classical scrapie (Fig. 1a and c). WB of H800 with P4 revealed a very faint low molecular mass PrP^{Sc} band (Fig.1b), estimated as ~8kDa, reminiscent of atypical scrapie and absent with 6H4 (Fig.1d). Suspecting degradation of protein in storage, we attempted to reproduce the pattern using different sheep brain samples which had been similarly stored but found only classical scrapie patterns (not shown). H800 was therefore classed as unusual.

In the second archive (NPU Cheviots), of the sheep that died before 1980, a single animal (13x85) with P4 showed PrP^{Sc} with a clear additional low molecular mass band estimated as ~7-9 kDa (Fig. 1e and g) which was absent with 6H4 (Fig. 1f). This was a female Cheviot (AF_{141}RQ/AF_{141}RQ), which was born in 1966 and died at 6 years of age in 1972. It had been challenged with experimental scrapie (details below) and was recorded in 1972 as having positive clinical signs of scrapie with a "very unusual clinical syndrome" and with "widespread moderate lesions" in brain.

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157 We have no further written records and have no fixed tissue available to re-examine
158 the pathology in this animal.
159
160 Sheep 13x85 was one of a group of 11 animals which were used in a study of scrapie
161 strain interference and challenged subcutaneously, first with SSBP/1 scrapie and
162 secondly, after two years without clinical signs, with CH1641. Three (all
163 $AF_{141}RQ/AF_{141}RQ$), including 13x85, of the 11 sheep became clinically affected with
164 scrapie signs at 1224-1524 days after SSBP/1 challenge and 500-800 days after
165 CH1641 challenge. Only one (13x69) of the other two scrapie affected sheep had
166 stored frozen tissue allowing a comparison by WB however this sheep showed a
167 classical scrapie-like PrP^{Sc} pattern without the ~7-9kDa molecular mass band seen
168 with 13x85 (Fig.1g). For comparison, WB of both CH1641 and SSBP/1 are also
169 shown (Fig. 1h and i). While PrP^{Sc} from SSBP/1 is recognised by both 6H4 and P4,
170 CH1641 shows much reduced staining with P4 compared with 6H4 which also reveals
171 a lower (~19kDa) unglycosylated band than is seen with natural scrapie or SSBP/1.
172
173 **Strain typing of candidate atypical scrapie cases by mouse bioassay.**
174 *(a) non transgenic inbred mice*
175 Because of the unusual clinical signs (unfortunately not recorded) in sheep 13x85,
176 transmissions were set up in 1972 to a wide range of inbred mouse lines but never
177 previously published. The results from RIII, C57/Bl(*Prnp*^{a} mice) and VM(*Prnp*^{b})
178 (Table 1) show 100% attack rate and mean incubation periods of 267±26, 297±25 and
179 468±73 days respectively. This was noted at the time as surprising as concurrent
180 transmissions from SSBP/1, CH1641 and natural classical scrapie from an NPU flock
181 VRQ/VRQ sheep (47x79) gave much lower attack rates and longer incubation periods

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(>480 days) (Table 1). Results from more recent atypical scrapie negative transmissions, including that from sheep 51x45, to the same mouse lines are also shown.

b) transgenic mice, tg338

We did not attempt additional strain typing in wild type mice as atypical scrapie, and frequently also classical scrapie, does not transmit well to non-transgenic mice (see Table 1). In consequence, the scrapie cases selected for further study (L4824, H800 and 13x85) were transmitted to tg338 mice, which express the ovine VRQ allotype (Le Dur *et al.*, 2005), and compared with classical and atypical scrapie controls, also in tg338 mice, including L4823 as a concurrent flock-mate control for L4824. We were not able to perform such transmissions with the similar control for 13x85 (13x69) because no sterile material suitable for bioassay was available.

Incubation period results are shown in Table 2 and summary Table 3. The classical scrapie control (68x81) had a very long (VL) incubation period of 584 ± 57 days (mean ± standard deviation). In contrast, SSBP/1, which is a "rapid strain" in tg338 mice, gave the expected short (S) incubation period of 76 (±7) days. CH1641 and the two atypical isolates, Scr2 and 51x45, all gave clinically positive mice with medium (M) incubation periods of 157 (±3), 191 (±46) and 175 (±23) days respectively. The unusual cases L4824 and 13x85 also gave M incubation periods of 173 (±13) and 135 (±14) respectively, although 13x85 gave an attack rate of <50%. H800 also had a low attack rate (<50%) and a wide range of M incubation periods (276 days, ±113) and L4823 produced very low numbers (2/11) of affected animals, with long (L) and very long (VL) incubation periods of 364 and 572 days.

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Vacuolation profiles in the clinically affected mice are illustrated in Fig.2. The natural classical scrapie control (68x81, Fig. 2a) showed a fairly flat lesion profile similar to CH1641 (Fig.2c) and arbitrarily designated as type B1 in summary Table 3. SSBP/1 (Fig. 2b) showed more vacuolation in regions G4 and G5 (type B2 in Table 3). The two atypical scrapie controls, Scr2 and 51x45 gave very similar and characteristic lesion profiles (Fig. 2d and 2e respectively, type A in Table 3), particularly striking in the damage caused in the white matter area W3 (basal cerebral peduncle) as expected (Griffiths *et al.*, 2010). Of the unusual scrapie transmissions, the vacuolar profile of L4824 (Fig. 2f) was very similar to that of atypical scrapie (type A), H800 (Fig. 2g) resembled classical scrapie (type B1) and 13x85 (Fig. 2h) had similarities to atypical scrapie in white matter damage but in grey matter looked different from any of the other tested strains (designated as mixed type in Table 3). L4823 produced only 2 clinical cases so no lesion profile could be produced for direct comparison; however the lesion damage patterns from each individual clinical mouse are shown in Fig.2i. L4823 mouse 4, incubation period 572d, (L4823/4) showed pathology resembling natural scrapie (type B1) whereas mouse 9, incubation period 364d, (L4823/9) has a distinct pattern very like atypical scrapie (type A).

Immunohistochemistry on tg338 mouse transmissions

With small variations in magnitude, mice within each inoculation group showed indistinguishable patterns and distribution of PrP^{d} accumulation (identical with 2G11 and SAF84), except for the two positive L4823 mice, as detailed below (Table S2 and Figs. 3 and 4). Mice infected with either of the two atypical scrapie controls Scr2 (Fig

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3a) and 51x45 (not shown) and L4824 (Fig 3c) had prominent, multifocal and bilateral coalescing PrP^d aggregates in thalamus which in some mice had the appearance of non-vascular plaque-like deposits. In the most severe cases, those coalescing accumulations were present also in neighbouring areas of the parietal cerebral cortex. Fine particulate PrP^d deposits were also found in the same locations, while other brain areas appeared devoid of PrP^d. Intracellular deposits were inconspicuous. Vacuolation was very severe (Table S2) particularly in white matter tracts of thalamus and midbrain, but was also present in cerebellum (grey and white matter) and other brain areas. This IHC pattern is designated type A in summary Table 3.

The IHC patterns in the classical scrapie control 61x81 (Fig 3b); H800 (Fig 3d) and mouse L4823/4, Fig.3e) were in contrast dominated by vascular and non-vascular PrP^d plaques located at the injection site and in subependymal and subpial areas throughout the brain, without involvement of the thalamus. Moderate fine particulate PrP^d deposits and mild to moderate intracellular PrP^d aggregates were observed in vestibular nuclei, habenula, hypothalamus, midbrain and cerebral cortex. In some mice intracellular aggregates were also seen in obex and deep cerebellar nuclei. Vacuolation was mild to moderate (Table S2). This IHC pattern is designated as type B1 in Table 3.

SSBP/1 and CH1641 were characterized by absence of plaques or coalescing PrP^d deposits, mild to moderate particulate and intracellular aggregates (Fig. 3f) with very little vacuolation (Table S2), designated as IHC type I in Table 3.

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Mice from the 13x85 transmission and mouse L4823/9 interestingly showed mixed features. With 13x85, PrP^{d} accumulation (Fig. 4a and 4b) was very similar to atypical scrapie (bilateral coalescing PrP^{d} deposits in thalamus, severe vacuolation, Table S2). However, like SSBP/1 and CH1641, particulate deposits were more prominent and widespread, also having intracellular (intraneuronal and intramicroglial) PrP^{d} accumulation in midbrain, habenula, hypothalamus, striatum and cerebral cortex (IHC type A+I in Table 3). L4823/9 showed a mixture of features of atypical scrapie (coalescing PrP^{d} deposits in thalamus, prominent vacuolation, Fig 4c) and of classical scrapie (PrP^{d} plaques and particulate deposits, Fig 4d; IHC type A+B1 in Table 3).

Western blotting of mouse transmissions

Mouse brain samples from clinical and pathology positive animals were subjected to WB to examine the patterns of PrP^{Sc} (Fig. 5). L4824 and 51x45 PrP^{Sc} both showed low molecular mass doublet bands (~7-9kDa) not detected by 6H4 (Fig. 5a and c, lanes 2 and 3) as expected from atypical scrapie. PrP^{Sc} from H800 (Fig. 5a and c, lane 7) has similar staining properties to classical scrapie (Fig. 5a and c, lanes 1 and 9) and SSBP/1 (Fig. 5b and d, lanes 4-6) showing a three-banded pattern with ~21kDa lower molecular mass band and stronger staining with P4 than with 6H4. Even with over exposure (not shown) there was no sign of the faint ~8kDa band seen in the original sheep sample. PrP^{Sc} from 13x85 showed features of both atypical and classical scrapie on the Western blots. Initially (Fig. 5a and c, lane 5) the pattern resembled classical scrapie but by increasing the loading (Fig. 5e and h, lane 1 and Fig. 5f, lane 2), a band at ~7 kDa was clearly visible with P4 but not with 6H4. CH1641 also had different staining with the two antibodies (Fig. 5g and i, lanes 4 and 8). With 6H4 the lower molecular mass band (~19kDa) typical of CH1641 in sheep was reproduced in

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the tg338 mice however with P4 this band was not stained and instead a ~21kDa band was visible, unlike PrP^{Sc} in CH1641 affected sheep which is largely unlabelled by P4 (Jeffrey *et al.*, 2006a).

The two clinically positive mice from the L4823 transmissions are shown in Figs. 5g and i with each mouse (L4823/4 and L4823/9) showing different patterns. L4823/4 was similar to classical scrapie with the typical three-banded pattern showing more intense staining with P4 than 6H4 (Fig. 5g and i, lane 3). L4823/9 also showed this pattern but in addition, with P4, had the low molecular mass doublet at ~7-9kDa characteristic of atypical scrapie (Fig. 5g and i, lanes 5 and 10). Negative control mice, tg338 with no inoculation, showed no staining whatsoever with 6H4 or P4 (not shown).

A summary of the findings for each inoculum and our conclusions on strain identity is given in Table 3.

Discussion

In this study of sheep samples dating back to the 1960s, ~1850 animals from our combined UK and NPU Cheviot archives were searched for signs of atypical scrapie using a screening process designed to establish the earliest date at which it had occurred but not expected to find every case. The characteristics of atypical scrapie are well established (Tranulis *et al.*, 2011) but it is not clear whether there is a single atypical strain (eg Nor98) or several different similar strains. WB patterns and pathology can vary from isolate to isolate however atypical scrapie transmissions suggest that this results from unknown host factors rather than strain variability

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(Arsac *et al.*, 2009; Gotte *et al.*, 2011). Here we have used the term atypical scrapie to encompass both possibilities, either a single entity or a group of similar strains.

We used IHC, Western blotting and *PRNP* genotyping to select sheep samples for TSE strain typing in tg338 mice in which classical natural scrapie, SSBP/1, CH1641 (designated in Table 3 as Classical types 1,2 and 3 respectively) and atypical scrapie can be distinguished using details of pathology and differential antibody staining. Tg338 mice, expressing the sheep VRQ allele, have been used successfully to analyse sheep scrapie cases, both classical and atypical, in a way which was not previously very easy due to the difficulties of transmission to wild type mice (Andreoletti *et al.*, 2011; Le Dur *et al.*, 2005; Thackray *et al.*, 2012). Results were however compared with wild type mouse transmissions, where available.

In Table 3 we have presented our conclusions on strain identity. From our UK archive, one sheep, H800, initially thought to be atypical scrapie because of a faint ~8kDa band on Western blot, resembled classical scrapie by lesion profiling, IHC and WB on transmission to mice. Moreover its genotype (VRQ/AF_{141}RQ) is rarely affected by atypical scrapie and it is therefore more likely that the low molecular mass band in Western blots from the original sheep was the result of tissue degradation in storage. Clearly the presence of ~7-9kDa protein bands in PrP^{Sc} preparations needs interpreted with caution.

L4824, which died in 1988, had characteristic histopathology and was of a *PRNP* genotype (AF_{141}RQ/AL_{141}RQ) common for atypical scrapie. On transmission to tg338 mice, the lesion and IHC profiles were very similar to the two atypical scrapie

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controls. Both sheep and mouse WB patterns were similar in having a doublet band at 7-9kDa recognised by P4 and not 6H4, a characteristic of atypical scrapie. L4824 had a companion case from the same flock (L4823) which had several features consistent with classical scrapie including IHC and WB pattern and was selected for transmission as a convenient concurrent control. Surprisingly, on transmission to tg338 mice, however, one of the two clinically affected mice had pathology and WB pattern consistent with classical scrapie (L4823/4) while the other (L4823/9) had a mixture of classical and atypical scrapie features. One possible explanation is that the sheep had both classical and atypical scrapie with the features of the latter masked by those of classical scrapie (IHC and vacuolation) and/or lost due to degradation on storage (WB). Once transmitted to tg338 mice, which are highly sensitive to very low titres of atypical scrapie (Andreoletti *et al.*, 2012), a single mouse (L4823/9) may have detected the atypical scrapie agent against a background of equally low effective titres of classical scrapie.

The 1972 case from the NPU Cheviot flock (13x85) is also problematic. The sheep 13x85 was born in 1966 and formed part of a group in which mixed infection was attempted with two strains of experimental scrapie in separated injections: SSBP/1 and then CH1641. Clinical disease occurred with an incubation period that we now know could have resulted from either strain in $AF_{141}RQ/AF_{141}RQ$ sheep (Houston, Goldmann and Hunter, in preparation). The super-infection with CH1641 could have been blocked by an already replicating SSBP/1 or could have resulted in a mixed experimental infection, layered on top of, or combined with, atypical scrapie which could have arisen at any point in the animal's life. Sheep 13x85 developed clinical signs described in 1972 as unusual and subsequent transmission to wild type mice was

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very efficient and more aggressive than CH1641, SSBP/1, NPU classical scrapie or atypical scrapie alone. No lesion profiles are available for comparison from that original mouse transmission however our findings from tg338 mouse transmissions (IHC and WB) suggest that 13x85 was an atypical scrapie case, superinfected with either or both of CH1641 and SSBP/1. The question is: did this sheep develop atypical scrapie naturally or did it emerge as a result of infection with two experimental sources of classical scrapie? We believe that the latter is unlikely since another sheep subjected to the same combined infection (13x69) did not develop features of atypical scrapie.

Natural mixed atypical/classical infection of single sheep has been reported elsewhere (Mazza *et al.*, 2010). Intriguingly, recent PrP^{Sc} molecular studies have found evidence of natural mixed infections including CH1641-like strains in sheep (Langeveld *et al.*, 2014) and experimental inoculation studies have suggested that the phenotype of atypical scrapie can in some instances change into that of CH1641 during passage in sheep (Simmons *et al.*, 2015) . It is therefore more likely that natural TSE infections of ruminants involve mixtures of strains, or have a more fluid identity, rather than the single strains normally used in lab studies. Combinations of infections have been implicated in the development of novel strains with altered host range in lentivirus infections of ruminants (Minardi da Cruz *et al.*, 2013) and such combinations of strains and types of virus/bacteria/prions, particularly in persistent infections, are more likely to represent the real-life situation in the field and could favour the emergence of more highly virulent disease causing agents. Whereas viruses can recombine their genetic material to form new strains, the concept of the

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prion being free from any nucleic acid makes it challenging, but also important, to understand how mixed prion strains interact.

Sheep 13x85 provides the earliest evidence to date for atypical scrapie in sheep. The finding of cases occurring up to 26 years prior to the original Nor98 animals lends weight to the hypothesis that atypical scrapie is not a newly emerging TSE but was discovered simply as a result of increased surveillance. Its risk for human health following consumption of sheep products remains unknown but the length of time atypical scrapie has existed in sheep, supports the idea that it represents no additional risk.

Methods

Sheep tissue archives

Our archive of scrapie affected and healthy sheep tissues dates back to the 1960s and originated at the Neuropathogenesis Unit (NPU) but is now held at The Roslin Institute. For this study we divided the archive into two: those from around the UK (UK archive) and those from our own Cheviot flock (NPU archive). The UK archive samples (n ~350) were from sheep, scrapie affected and healthy, from various locations and with death dates 1964-2004. One UK animal, a Dorset Horn (377J) that died in 1989, has already been reported by us to have had atypical scrapie (Bruce *et al.*, 2007). In the NPU archive, at the time this study was carried out, there were approximately 1500 sheep represented, with death dates 1966-2005, but the selection process (see below) reduced this number to ~200 samples for further study. Extensive records cover the foundation of the flock in 1960 to date and studies of its endemic classical scrapie have been reported (Hunter *et al.*, 1996; Matthews *et al.*, 1999;

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Matthews *et al.*, 2001; Redman *et al.*, 2002; Woolhouse *et al.*, 1999) along with details of a case of atypical scrapie from 2001 (Foster *et al.*, 2008).

All information, however limited, that was available in original records was collated, eg clinical signs, *PRNP* genotype, age at death, flock location, haematoxylin and eosin (H&E) stained vacuolation pathology results. The condition of the samples was highly variable due to long term storage. Tissues suitable for immunohistochemistry (formal saline fixed or wax blocks) and/or for Western blotting (frozen) and mouse transmission (sterile and frozen) were available but not all three for every animal.

Search for atypical scrapie in sheep samples: selection criteria

The selection process, searching for the characteristics of atypical scrapie (Table S1), used three lines of processing (Fig. S1) after removing samples which were too degraded for DNA analysis or too dried out for immunohistochemistry (IHC). Firstly, frozen samples were *PRNP* genotyped, mostly excluding genotypes associated with classical scrapie and PrP^{Sc} protein analysis was carried out on the relatively few samples which remained. Secondly, fixed tissues suitable for IHC were examined for signs of atypical scrapie pathology. At the final stage of the process, samples were considered for strain typing in mice if atypical scrapie signs had been found by IHC and/or WB. A third line of selection involved animals, regardless of genotype, which had records from the time of death suggesting unusual clinical/pathology signs. Only if the latter also had positive atypical signs from WB and/or IHC were these sheep considered for mouse strain typing.

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Genotyping

Genomic DNA was extracted from blood or tissue samples using the Qiagen DNeasy Blood & Tissue Kit, following the manufacturer's instructions. *PRNP* genotyping was performed on PCR-amplified DNA fragments as described (Hunter *et al.*, 2012). Genotypes are presented here in either the three codon (136, 154, 171) format (eg VRQ/ARQ) or, for the ARQ allele on which codon 141 can vary, a four codon format is used if known (eg $AL_{141}RQ/AF_{141}RQ$). All other alleles in this report were L_{141}.

Immunohistochemistry

Our immunohistochemistry (IHC) methods do not result in detection of PrP^{C} but as no proteinase K (PK) is used in the process, the PrP protein detected by IHC is referred to here as disease-associated PrP (PrP^{d}) (Gonzalez *et al.*, 2005). The term PrP^{Sc} is here reserved for the protein detected in WB of PK treated samples.

In certain sheep cases paraffin embedded brain tissues were available from previous routine H&E investigations. These were re-examined with current immunohistochemistry (IHC) techniques for the detection of PrP^{d} (Foster *et al.*, 2001; Jeffrey *et al.*, 2006b) using the BG4 anti-PrP monoclonal antibody at 1µg/ml (TSE Resource Centre, The Roslin Institute). Following transmission in tg338 mice, formalin-fixed, wax-embedded murine brain tissues were examined as follows. For antigen retrieval, 4 µm tissue sections were immersed in formic acid for 5min at 20°C followed by autoclaving in 0.2% citrate retrieval solution (pH 6.8) at 121°C for 30 min. After washing in tap water and quenching in hydrogen peroxide (3% in methanol) for 20 min, tissue sections were blocked with the MOM kit liquid protein concentrate solution (Vector Laboratories, Peterborough, UK) at a dilution of 1:20 for

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60 min and incubated overnight at 23°C with 1µg/ml 2G11 (Novus Biologicals, Littleton, USA) or 0.2µg/ml SAF84 (SPI bio, Montigny Le Bretonneux, France) which are both mouse monoclonal antibodies recognising amino acid residues 153-158 (Thuring *et al.*, 2004) and 166-172 (Jacobs *et al.*, 2011) of ovine PrP, respectively. The subsequent steps of the IHC procedure were performed using the immunoperoxidase Elite ABC kit (Vector Laboratories). Sections were then counterstained with Mayer's haematoxylin.

Biochemical detection of PrP^{Sc}

Where sufficient tissue was available selected samples were subjected to PrP^{Sc} analysis by Western blot as described (Jeffrey *et al.*, 2006a; Stack, 2002) with a non-stringent proteinase K concentration of 50µg/ml. Samples were run on 16% Tris-glycine gels (Invitrogen, Paisley, UK) and immunoblotted onto polyvinylidene difluoride (PVDF) membranes. For detection of PrP^{Sc}, two monoclonal antibodies were used: 6H4 (Prionics, Schlieren, Switzerland) 2mg/ml, diluted 1:5000, and P4 (R-Biopharm, Darmstadt, Germany) 1mg/ml, diluted 1:2500 followed by "visualization" with a chemiluminescence substrate (Roche, Lewes, UK) and Lumi-film (Roche). P4 labelling reveals the ~7-9kDa protein band(s) characteristic of atypical scrapie and if these low molecular mass bands were present, the membrane was stripped of antibody using Restore (Thermo Fisher Scientific, Loughborough, UK) and re-probed with 6H4 which binds to, and reveals, the 18-30kDa bands but not the ~7-9kDa band(s).

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Strain typing in mice

Study of The Roslin Institute data archive revealed that one of the unusual cases, 13x85, had been transmitted to wild type inbred mice (RIII, C57 and VM) in 1972. These historical results, which have never been published, are presented here along with transmissions of SSBP/1, CH1641, atypical scrapie (Scr2 and 51x45) and a VRQ/VRQ NPU classical scrapie case (47x79), which were carried out between 1989 and 2002. NPU scrapie has been transmitted to mice many times from different sheep, and the older results are very similar to those from more recent transmissions. All of this historical data is now part of The Roslin Institute data archive and all of the older studies were approved by the relevant ethics committees and conducted under the licencing appropriate at the time. The infection procedures, daily observation of animals and clinical assessments we use now were first established by A.G. Dickinson in the 1960s and as a result the experiments are directly comparable. Lesion profiles for the 13x85 wild type mouse transmissions have been lost however.

Because atypical scrapie does not transmit to wild type mice, brains from the three cases of interest plus four control cases were strain typed by intracerebral injection of 20µl of 10% brain homogenate into tg338 mice after pre-screening for bacterial contamination. The experiments were approved by The Roslin Institute Ethics Committee and were carried out under UK Home Office personal and project licences.

Control inoculations included (1) three classical scrapie types, two showing 21kDa unglycosylated PrP^{Sc} band on Western blots (SSBP/1, which produces a very short incubation period in tg338 mice, and an NPU natural scrapie case of VRQ/VRQ

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genotype, 68x81) and a sheep (J2916) affected by CH1641, a classical scrapie with a 19kD unglycosylated PrP^{Sc} band and (2) two confirmed atypical scrapie cases: Scr2 (Bruce *et al.*, 2007) and 51x45 (Foster *et al.*, 2008). We also included a flock-mate of one of the candidate atypical cases as a concurrent control (L4823).

Mice were observed daily and culled when clear clinical signs of scrapie or other intercurrent disease were noted. Each mouse brain was examined (blinded to the inoculum) for vacuolation in H&E sections and a lesion profile generated as described previously (Fraser & Dickinson, 1968). Incubation periods were calculated as an average of time between inoculation and death for all clinically positive and vacuolation positive mice. For some purposes incubation period mean durations are presented in the following format: S (short, mean <100days), M (medium, mean 100-200 days), L (long, mean 200-400) or VL (very long, mean >400 days). Survivors were those mice that lived longer than the earliest positive case.

In addition, for each of the ovine inocula, groups of 4 clinically affected mice were examined by IHC, with the exception of those inoculated with L4823 (see Table 1) for which only two mice developed neurological signs. The magnitude of PrP^{d} accumulation was scored (blinded to the inoculum) as follows: 0 (absent) to 3 (severe) in brain areas, (1): frontal cortex, corpus striatum, basal ganglia; (2): temporo-parietal cortex, hippocampus, thalamus, hypothalamus; (3): midbrain; (4): cerebellar cortex, deep cerebellar nuclei, pons; (5): medulla oblongata. The PrP^{d} types observed were intracellular (intraneuronal and intraglial combined), fine particulate, coalescing and plaques (vascular and non-vascular combined). The final score was an average of the different PrP^{d} types for each group. Since differences in the severity of vacuolation

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were observed in the brains, mice were given an overall score from 0 to 3 which gives a parallel and more general measure of vacuolation than the lesion profiles (see above).

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		Mouse lines					
Inoculum	Transmission date	RIII		C57		VM	
		Attack rate* Ns/Ni	Incubation period† in days, mean (SD)	Attack rate Ns/Ni	Incubation period in days, mean (SD)	Attack rate Ns/Ni	Incubation period in days, mean (SD)
13x85 unusual scrapie	1972	6/6	267 (26)	6/6	297 (25)	5/5	468 (73)
SSBP/1	1971	2/4	486, 505	5/5	517 (3)	5/5	506 (47)
CH1641	1989	7/18	481 (66)	3/18	682, 689, 764	3/18	717, 717,683
47x79, classical scrapie, natural	1994	0/23	NA	9/23	592 (83)	3/15	544,596, 604
Scr2ǂ, Atypical scrapie	1990	0/23	NA	0/13	NA	0/18	NA
51x45, Atypical scrapie	2002	0/23	NA	0/19	NA	0/24	NA

Table 1. Incubation periods in days (d) with standard deviation (SD) of transmissions of sheep brain in three different inbred mouse lines, carried out between 1971 and 2002. * numbers of clinical positive, vacuolation positive mice(Ns)/ number injected (Ni). †Mean incubation period with SD when >5 positive cases, otherwise individual mouse incubation periods given. ǂdata adapted from Bruce et al 2002. NA = not applicable

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Inoculum and/or sheep identity	Scrapie type	Origin (date of death)	Genotype	Attack rate* in tg338 mice Ns/Ni	Incubation period† in days, mean (SD)	Range or actual incubation period if <5 mice (days)
68x81	Classical 21kD, natural	NPU flock (2000)	VRQ/VRQ	12/12	584 (57)	482-677
SSBP/1	Classical 21kD, rapid	NPU (pre-1960)	Pooled clinical sheep brain (all VRQ encoding)	6/6	76 (7)	64-83
CH1641 (J2916)	Classical, 19kD	NPU (2000)	ARQ/AHQ	12/12	157 (3)	155-163
Scr2	Atypical	UK (1989)	AHQ/AHQ	10/11	191 (46)	165-310
51x45	Atypical	NPU flock (2001)	AHQ/AHQ	11/12	175 (23)	150-217
L4824	Unusual case	UK (1988)	AHQ/ARR	10/11	173 (13)	151-196
L4823	L4824 Flockmate	UK (1988)	VRQ/AL_{141}RQ	2/11	NA	364, 572
H800	Unusual case	UK (1977)	VRQ/AF_{141}RQ	5/11	276 (113)	163-462
13x85	Unusual case	NPU (1972)	AF_{141}RQ/AF_{141}RQ	5/11	135 (14)	125-160

Table 2. Origins and characteristics of the sheep inocula and tg338 mouse transmission features (attack rate and incubation period).

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* numbers of clinical positive, vacuolation positive mice (Ns)/ number injected (Ni). † Mean incubation period with SD when >5 positive cases, otherwise individual mouse incubation periods given. NA= not applicable

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Inoculum	Sheep, source of inoculum			WT mice, three lines*		Tg338 mice						Conclusion on strain type
	IHC	WB, lowest molecular mass band (kDa)		Attack rate	Incubation period (d)	Attack rate	Incubation period (d)	Vacuolation Profile type	IHC type	WB, lowest molecular mass band (kDa)		
		6H4	P4							6H4	P4	
Classical scrapie: 68x81(WT mice) and 47x79 (tg338)	Class	21†	21	0-39%	VL	100%	VL	B1	B1	21	21	Classical (1)
SSBP/1	Class	21	21	50-100%	VL	100%	S	B2	I	21	21	Classical (2)
CH1641 (J2916)	Class	19	X	17-39%	VL	100%	M	B1	I	19	21	Classical (3)
Scr2	Atyp	X	7-9	0	NA	91%	M	A	A	X	7-9	Atypical
51x45	Atyp	X	7-9	0	NA	92%	M	A	A	X	7-9	Atypical
L4824	Atyp	21	7-9	ND	NA	91%	M	A	A	X	7-9	Atypical
L4823	Class	21	21	ND	NA	18%	L	A+B1	A+B1	21	7-9	Mixed atypical and classical (1)
H800	ND	21	~8	ND	NA	45%	L	B1	B1	21	21	Classical (1)
13x85	ND	21	7-9	100%	L	45%	M	Mixed, A + another strain: B1 and/or B2	A+I	21	7-9	Mixed atypical and classical (2/3)

Table 3. Summary of all data from sheep brain samples and transmissions to Wild Type mice (WT) and transgenic mice (tg338) and conclusions on strain typing. * Summary of data from three mouse lines abstracted from Table 1. Incubation period and immunohistochemistry

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designations as defined in text. IHC: immunohistochemistry; WB: Western blot; † lowest molecular mass (kDa) of PrP^{Sc} labelled by either of the antibodies 6H4 or P4; Attack rate as defined in Tables 1 and 2; Class = classical scrapie; Atyp: atypical scrapie; ND: not done; NA: not applicable; X: nothing labelled on the Western blot.

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Figure legends

Fig. 1: Western blots of PrP^{Sc} from sheep brain of unusual scrapie cases and controls using antibodies P4 (a, b, e, g, h) and 6H4 (c, d, f, i). M= molecular mass markers (kDa). (a, c): lanes 1-4, L4823, serial 1:3 dilutions; lanes 5 and 6, L4824, serial 1:3 dilutions; (b, d): lane 1-3, H800, serial 1:3 dilutions. (e): lane 1, 13x85; lane 2 and 3, two concurrent natural scrapie sheep; lane 4, 13x85, 1:3 dilution; lane 5 and 6, concurrent natural scrapie sheep, 1:3 dilutions. (f): lane 1, unrelated sample; 2, SSBP/1; 3, 13x85. (g): lanes 1, 3 and 5, natural scrapie controls; lane 2, 13x69; lane 4 13x85. (h, i): lanes 1 and 4, CH1641; lanes 2 and 5, SSBP/1; lane 3, unrelated sample.

Fig. 2: Lesion profiles of unusual scrapie cases and controls in brain of clinically affected tg338 mice. Patterns of severity of vacuolation (with Standard Error bars) in nine grey matter areas (G1-G9: medulla, cerebellum, superior colliculus, hypothalamus, thalamus, hippocampus, septum, retrosplenial cortex and cingulate & motor cortex) and three white matter areas (W1-W3: cerebellum, superior cerebral peduncle, basal cerebral peduncle). (a) classical scrapie 68x81; (b) SSBP/1; (c) CH1641; (d) atypical scrapie, Scr2; (e) atypical scrapie 51x45; (f): L4824; (g): H800; (h) 13x85; (i) vacuolation damage in two individual clinically positive mice inoculated with L4823. Dotted line: mouse 4 (L4823/4), dashed line: mouse 9 (L4823/9).

Fig. 3: Immunohistochemical PrP^{d} features of tg338 mice. Thalamus from mice inoculated with (a) atypical scrapie (Scr2); (b): classical scrapie (68x81); (c): L4824;

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(d): H800; (e) L4823/9; (f) Dorsal motor nucleus of the vagus from mouse inoculated with SSBP/1. IHC with 2G11 or (f only) SAF84 with haematoxylin counterstaining.

Fig. 4: Immunohistochemical PrP^{d} features of tg338 mice inoculated with sheep brain. (a,b) thalamus from mice inoculated with 13x85, (c) corpus striatum and (d) deep cerebellar nuclei from mouse L4823/9. IHC with 2G11 and haematoxylin counterstaining.

Fig. 5: Western blots of PrP^{Sc} from brain of tg338 mice inoculated with unusual or control scrapie sheep brain using antibodies P4, (a, b, e, f, g) and 6H4 (c, d, h, i). M=molecular mass markers (kDa). Inocula: (a, c): lanes 1, 6 and 9, natural scrapie control (68x81); lane 2, L4824; lane 3, atypical scrapie control (51x45); lanes 4 and 8, CH1641(J2916); lane 5, 13x85; lane 7, H800; (b, d): lanes 1-3, 1:3 dilutions of atypical scrapie, Scr2; lanes 4-6, 1:3 dilutions of SSBP/1. (e): lane 1, atypical scrapie (51x45); lane 2, 13x85 loaded x6. (f): lanes 1 and 3, natural scrapie controls, lane 2, 13x85 loaded x6. (g, h): lanes 1, 4 and 11, natural scrapie control (68x81); lanes 2 and 6, CH1641 (J2916); lane 3, 7 and 8, single mouse L4823/4; lanes 5 and 10, single mouse L4823/9; lane 9, atypical scrapie control (51x45).

Fig S1: Flow chart of process of selecting candidate atypical scrapie cases from archive samples dating back to 1960s.

Fig. S2: Immunohistochemistry using BG4 of brain sections from scrapie case L4823 (a, c, e) and its unusual scrapie case flockmate L4824 (b, d and f). Obex (a) and (b), basal ganglia (c) and (d), cerebellum (e) and (f). Size bars=500µm.

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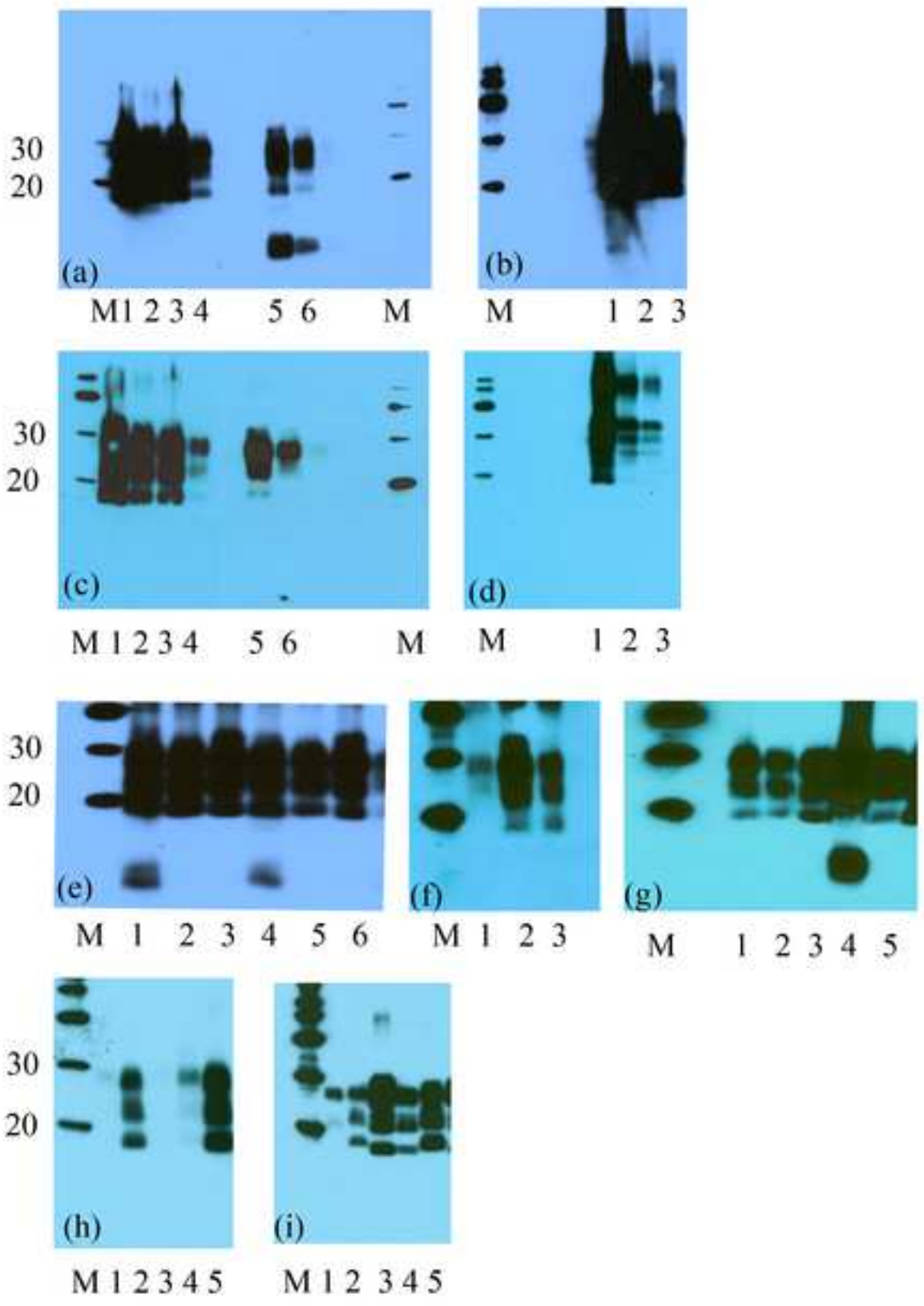

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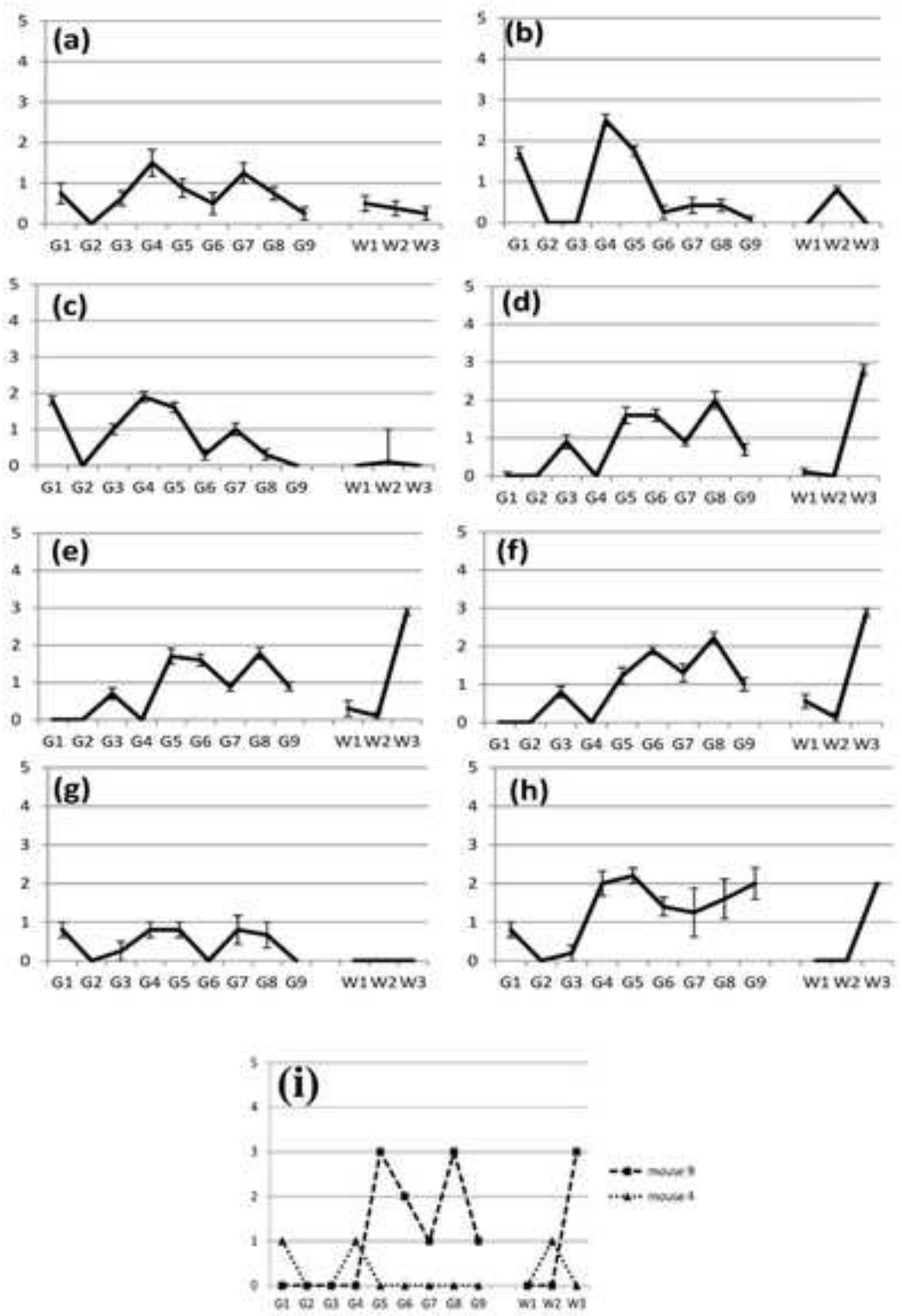

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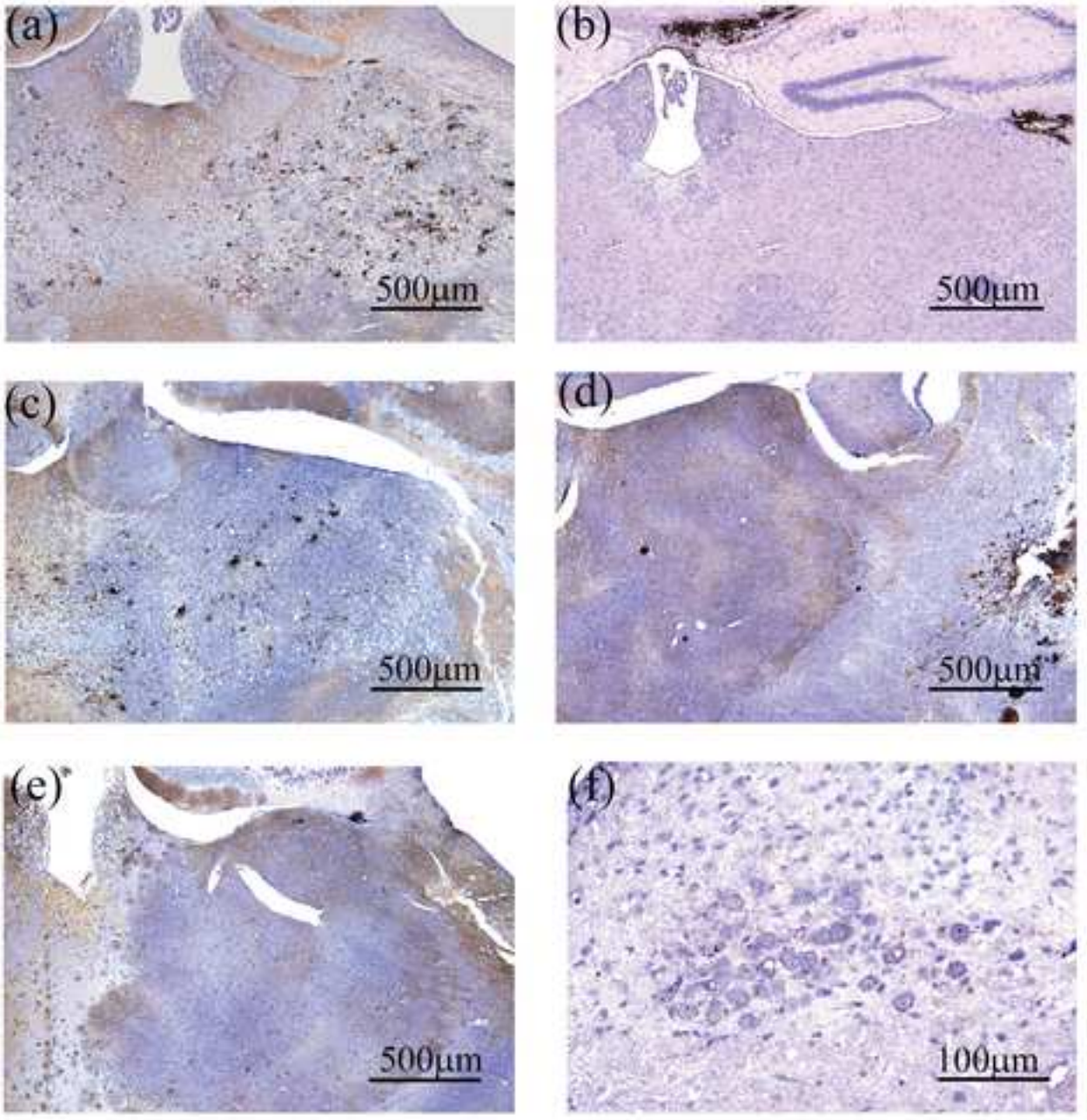

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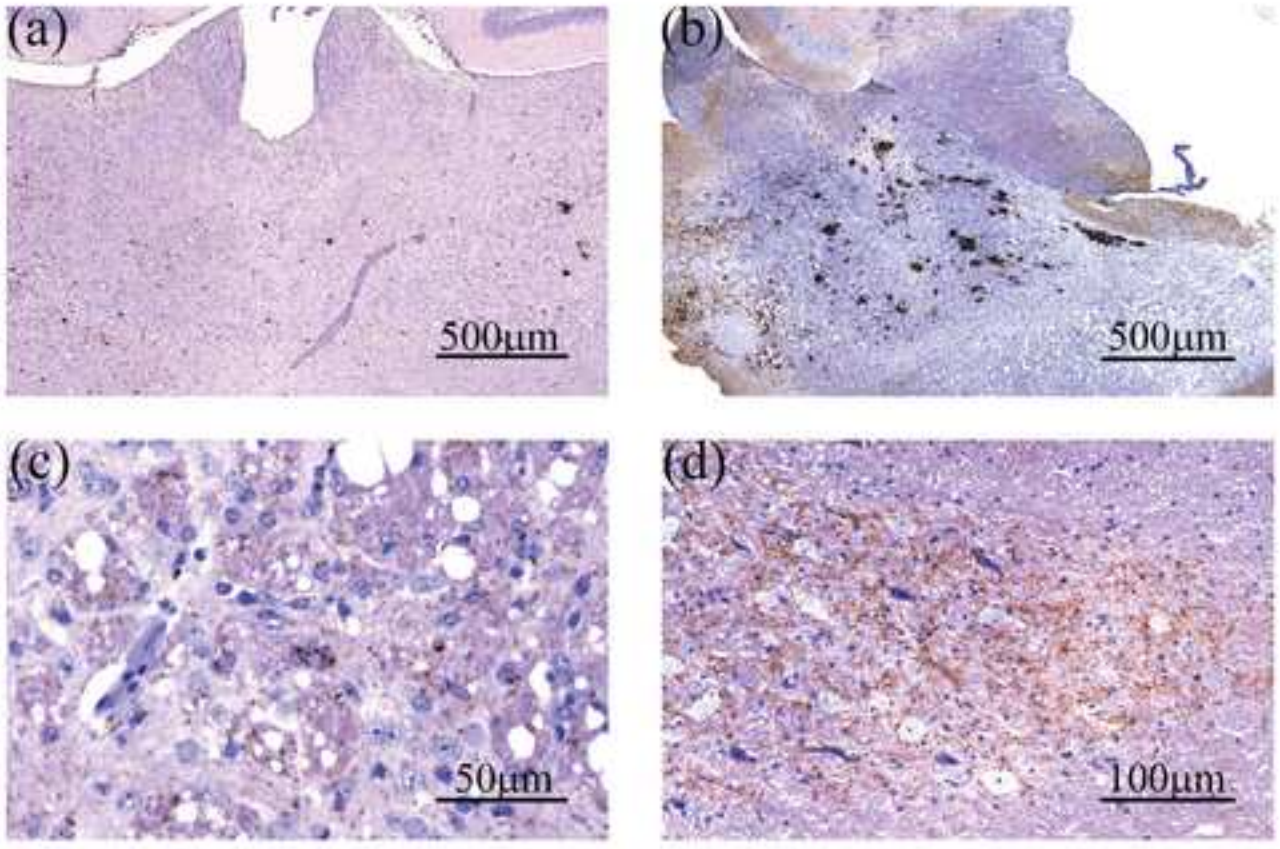

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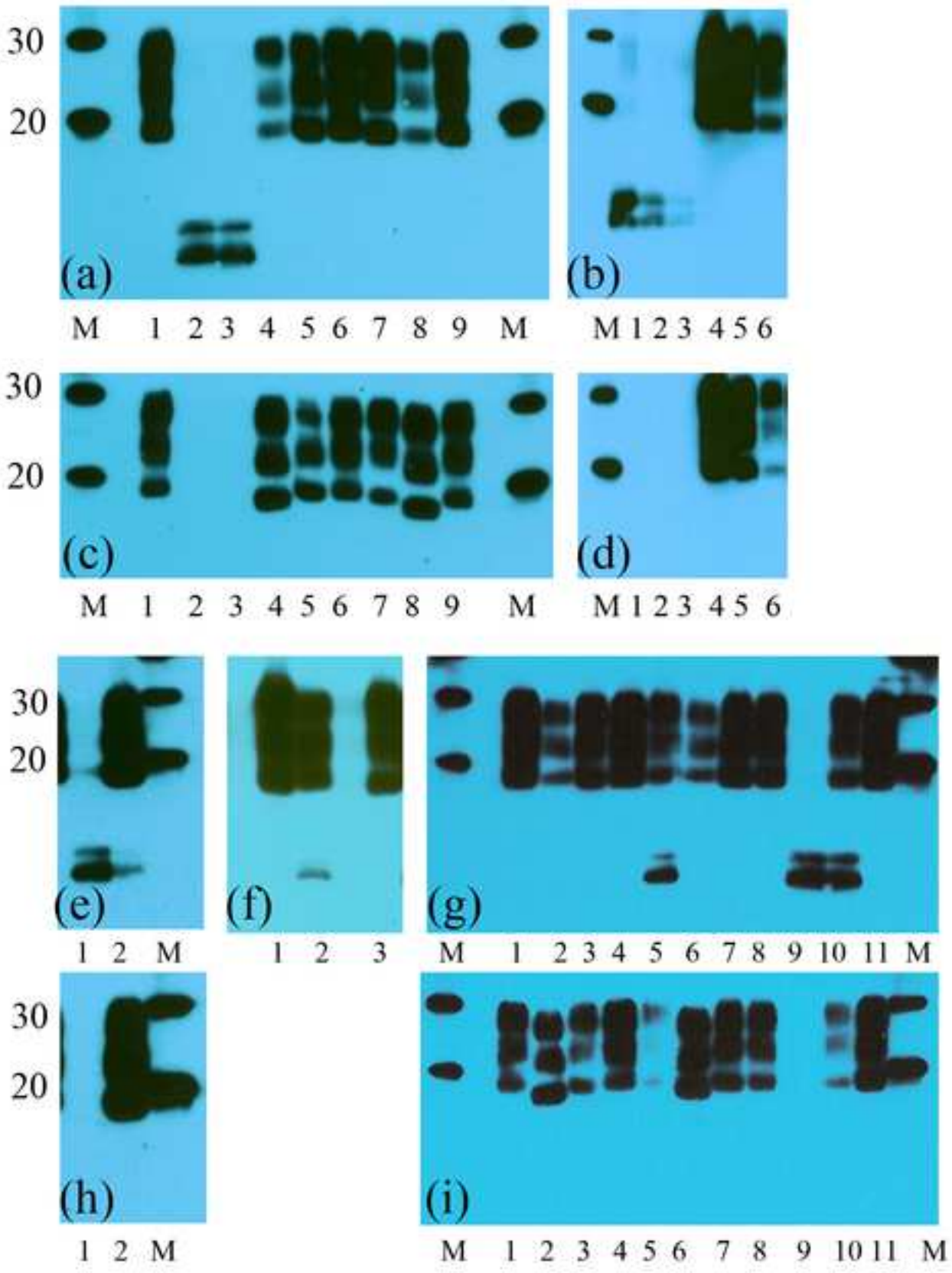

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